# STUDY OF THE QUANTITATIVE AND QUALITATIVE CHANGES IN CHITIN AT DIFFERENT STAGES OF RIPENING OF MUSHROOM FRUITING BODIES

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## Abstract

*We investigated the extraction of chitin with various solvents and changes in the amount of chitin and its molecular mass at different stages of maturity in the fruiting bodies of the fungi Laetiporus sulphureus, Tyromyces chioneus, Oudemansiella mucida, Lycoperdon perlatum, and Fomitopsis betulina. We extracted chitin from crushed mushroom fruiting bodies with 25% hydrochloric acid at 0°C. We characterised the molecular weight of reprecipitated purified chitin with a viscosimeter and Fourier-transform infrared spectroscopy. The mass of extracted chitin in all studied mushrooms initially increased from youth to maturity, and then decreased after ripening. At the same time, the molecular weight of chitin tended to increase.*

***Keywords:*** *chitin, changes in the amount and molecular mass, fungi, FT-IR spectra*

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# 1. Introduction

Chitin, a polymer of beta–1,4–*N*–acetylglucosamine (GlcNAc), is the main component of arthropod exoskeletons. It is also an important component of the cell wall of fungi: it provides the stiffness and shape of the cell walls. Chitin is a characteristic component of the fungal classes Zygomycetes, Ascomycetes, Basidiomycetes, and Deuteromycetes, but it is not found in Oomycetes [1, 2]. According to the literature, the chitin content in the fruiting bodies of different types of mushrooms is not the same.

There are significant difficulties in purifying chitin from raw materials, so in most studies researchers determine the chitin content with an indirect method. The chitin-containing material is hydrolysed with 6 N hydrochloric acid (HCl) at 106°C for 24 h. After neutralisation, the *N*–acetyl–D–glucosamine content is determined in the hydrolysate, which is used to calculate the chitin content [3, 4]. The disadvantage of this approach is the possibility of attributing mono– and oligosaccharides of *N*–acetyl–D–glucosamine in the mushroom material to chitin, leading to overestimation. In addition, this method cannot evaluate the changes in molecular mass that may occur during the maturation of fruiting bodies. Chitin extraction from fungal material faces significant difficulties because chitin in only soluble in a limited range of solvents, which also promote chitin hydrolysis. These solvents include strong mineral acids, some salts, and so-called ionic liquids [5, 6]. Most of them are aggressive or scarcely available.

In this study, we evaluated the chitin content in fruiting bodies of the mushrooms *Laetiporus sulphureus* (Bull.) Murrill; *Tyromyces chioneus* (Fr.) P. Karst; *Fomitopsis betulina* (Bull.) B.K.Cui, M.L. Han et Y.C.Dai; *Oudemansiella mucida* (Schrad) Höhn; and *Lycoperdon perlatum* Pers. We collected these fruiting bodies at different stages of ripening and examined the changes that occur during maturation.

# 2. Materials and Methods

## 2.1. Materials

Fruiting bodies of the mushrooms *L. sulphureus*, *T. chioneus*, *O. mucida*, *L. perlatum*, and *F. betulina* were collected on the outskirts of the city of Lviv mainly in the summer-autumn period. Immediately after collection, they were dried to a constant weight in a drying cabinet at 55°C.

Daily observations were made in the places where these mushrooms grew. Approximately 1 week after the appearance of fruiting bodies (week 1), they were harvested (young mushrooms). Then, 1 week later (week 2), the mature mushrooms were collected from the same place. Finally, the fruiting bodies of overripe mushrooms were collected at week 3. At the same time, the fruiting bodies were divided into young, mature, and overripe considering the time of their collection and by assessing their morphological state. The young *F. betulina* fruiting bodies were pure white, soft, and easy to cut with a knife. Ripe material was harder and it was difficult to cut it with a knife. An overripe fruiting body was woody and very difficult to cut with a knife. For *L. perlatum*, the fruiting bodies were divided based on maturity after assessing the colour of the inner part. Young fruiting bodies were pure white, older ones were yellowish, and old ones were grey.

Chitin from shrimp shells was purchased from Sigma Aldrich Co (St. Louis, USA) (Lot #SLBL9061V).

In the work, alkalis, acids and salts of analytical purity were used to assess the solubility of chitin.

### 2.2. Apparatus

The viscosity of chitin solutions was measured in 25% HCl at 4°C with a VPZh-4 Ubbehold viscosimeter (Soyuznauchpribor, Russia) with a 0.82-mm diameter capillary.

The Fourier-transform infrared (FT-IR) spectra of samples were recorded on a Spectrum Two spectrometer (PerkinElmer, USA) using a diamond universal attenuated total reflectance single reflection accessory. PerkinElmer Spectrum software was used to draw the spectra. The spectra (16 scans per spectrum) of the solutions were collected from 4000 to 400 $cm^{-1}$, with a spectral resolution of 4 $cm^{-1}$.

### 2.3. Methods

*2.3.1. Solubility of Commercially Available Shrimp Chitin*

First, 30–33 mg of finely ground shrimp chitin was placed in a 10 ml conical centrifuge tube. Then, 3.0 ml of solvent was added and the mixture was stirred constantly for 2 h at 15°C. After that, the solution was centrifuged and the absence or presence of sediment and the volume of undissolved chitin were noted.

*2.3.2. Extraction and Primary Purification of Chitin from Mushroom Material*

An exact weight of dried, crushed, and sifted (through a sieve with a diameter of 1 mm) fruiting bodies was placed in a flask with a ground glass stopper. Then 25% HCl cooled to 0°C was added in a ratio of 1:10. The mixture was placed in a refrigerator at 0–2°C for 14 h and stirred periodically. After extraction, the mixture was centrifuged for 10 min at 6000 *g*, the supernatant was filtered, a piece of ice was added, and it was carefully alkalinised with 1 M sodium hydroxide (NaOH) to pH 6.0–7.0. The chitin precipitate was collected by centrifugation, after which it was washed with distilled water until the pH was neutral. It was re-dissolved in a minimum volume of 25% HCl cooled to –20°C. The undissolved residue was removed by centrifugation, the supernatant was filtered, a piece of ice was added, and the solution was carefully alkalinised with 1 M NaOH to pH 6.0–7.0. The resulting chitin precipitate was collected by centrifugation; washed with distilled water until neutral; washed with ethanol, acetone, and diethyl ether; and dried in an oven at 50°C. The dried chitin residue was weighed on an analytical balance.

*2.3.3. Determination of the Molecular Weight Using a Viscometer*

The molecular mass of the resulting products was determined with a Ubbehold VPZh-4 viscometer (Soyuznauchpribor, Russia) with a capillary 0.82 mm in diameter, as described for chitosan [7], except that 25% HCl was used as the solvent. The viscosity was measured at 4°C in 25% HCl.

## 3. Results and Discussion

First, we examined the solubility of commercially available shrimp chitin (obtained from Sigma) in various solvents. This chitin has a high molecular weight (80–920 kDa) [8–10] and a high degree of acetylation (82%–98%) [8, 9] and therefore requires harsh conditions for dissolution. As reported in the literature, chitin obtained from mushrooms should have a lower molecular weight [6, 11]. Therefore, it should be more soluble than shrimp chitin. Following this logic, the solvent that dissolves shrimp chitin would also dissolve mushroom chitin. We found that only HCl at a concentration above 25%, 85% orthophosphoric acid ($H_3PO_4$), and a saturated solution of beryllium bromide ($BeBr_2$) completely dissolved shrimp chitin. In contrast to the results obtained by other researchers [11, 12], we could not dissolve the shrimp chitin from Sigma in a mixture of 8 wt.% NaOH/4 wt.% urea. Therefore, we did not use this solvent. Due to the high viscosity of the $H_3PO_4$ and $BeBr_2$

solutions, it would be very difficult to mix these substances with raw materials, a factor that would significantly increase the extraction time. Hence, we chose to use 25% HCl to extract chitin from mushroom fruiting bodies.

We determined the molecular weight of a solution of shrimp chitin dissolved in 25% HCl at a temperature of 0–4°C. The average molecular weight was 120 ± 20 kDa. This indicates a decrease in molecular mass by at least 3–5 times. Therefore, it is obvious that the data given in Table 1 concerning the extraction of chitin from the fruiting bodies of mushrooms are underestimated. However, due to the fact that we obtained these experimental data under the same experimental conditions, we believe that it is possible to judge the dynamics of changes in the amount of chitin in fruiting bodies.

The results of molecular weight determination with a viscometer are also shown in Table 1. In all cases there was an increase in the amount of chitin from young to mature mushrooms. After ripening and the fruiting bodies had stopped growing, there was a decrease in the amount of chitin. Based on our data, the dynamics of these changes are not the same for each mushroom species. This phenomenon could be explained by the slightly different conditions during their harvesting (we harvested raw materials at different times, when weather conditions differed). According to the literature, chitin biosynthesis is most influenced by cultivation conditions and the condition of the enzyme system of the mushroom species [13].

We found that the molecular weight of fungal chitin tended to increase as the fruiting bodies aged. However, chitin obtained by HCl extraction had a much lower molecular weight than chitosan obtained from the same material. In a previous study, we obtained chitosan from fruiting bodies of *Lactarius pergamenus*, *Polyporus squamosus*, and *Pleurotus ostreatus* by alkaline hydrolysis of chitin-containing material. Their molecular weight was 36–97 kDa [14]. It is possible that during extraction with concentrated HCl, even at a temperature close to zero, there is significant hydrolysis of chitin. Drawing a parallel with the determination of the molecular weight of shrimp chitin and chitosan obtained from it, the molecular weight of mushroom chitin given in Table 1 should be at least 3–5 times greater.

**Table 1.** The molecular weight and the content of chitin of the mushroom fruiting bodies depending on the degree of maturity.

| No | Mushroom | Chitin content (from the mass of dried raw materials) [%] | Molecular weight of chitin [kDa] |
|---|---|---|---|
| 1 | *Laetiporus sulphureus* | (1) 0.65<br>(2) 1.41<br>(3) 0.47 | (1) 6 ± 1.0<br>(2) 11 ± 1.0<br>(3) 15 ± 1.2 |
| 2 | *Fomitopsis betulina* | (1) 1.37<br>(2) 2.38<br>(3) 0.50 | (1) 16 ± 1.2<br>(2) 18 ± 1.2<br>(3) 19 ± 1.2 |
| 3 | *Tyromyces chioneus* | (1) 0.25<br>(2) 0.28<br>(3) 0.16 | (1) 17 ± 1.2<br>(2) 18 ± 1.2<br>(3) 19 ± 1.2 |
| 4 | *Lycoperdon perlatum* | (1) 3.21<br>(2) 3.80<br>(3) 2.12 | (1) 19 ± 1.2<br>(2) 24 ± 1.2<br>(3) 24 ± 1.2 |
| 5 | *Oudemansiella mucida* | (1) 1.33<br>(2) 1.48<br>(3) 1.26 | (1) 19 ± 1.2<br>(2) 20 ± 1.2<br>(3) 21 ± 1.2 |

*Note.* (1) young fungi; (2) ripe fungi; (3) overripe fungi.

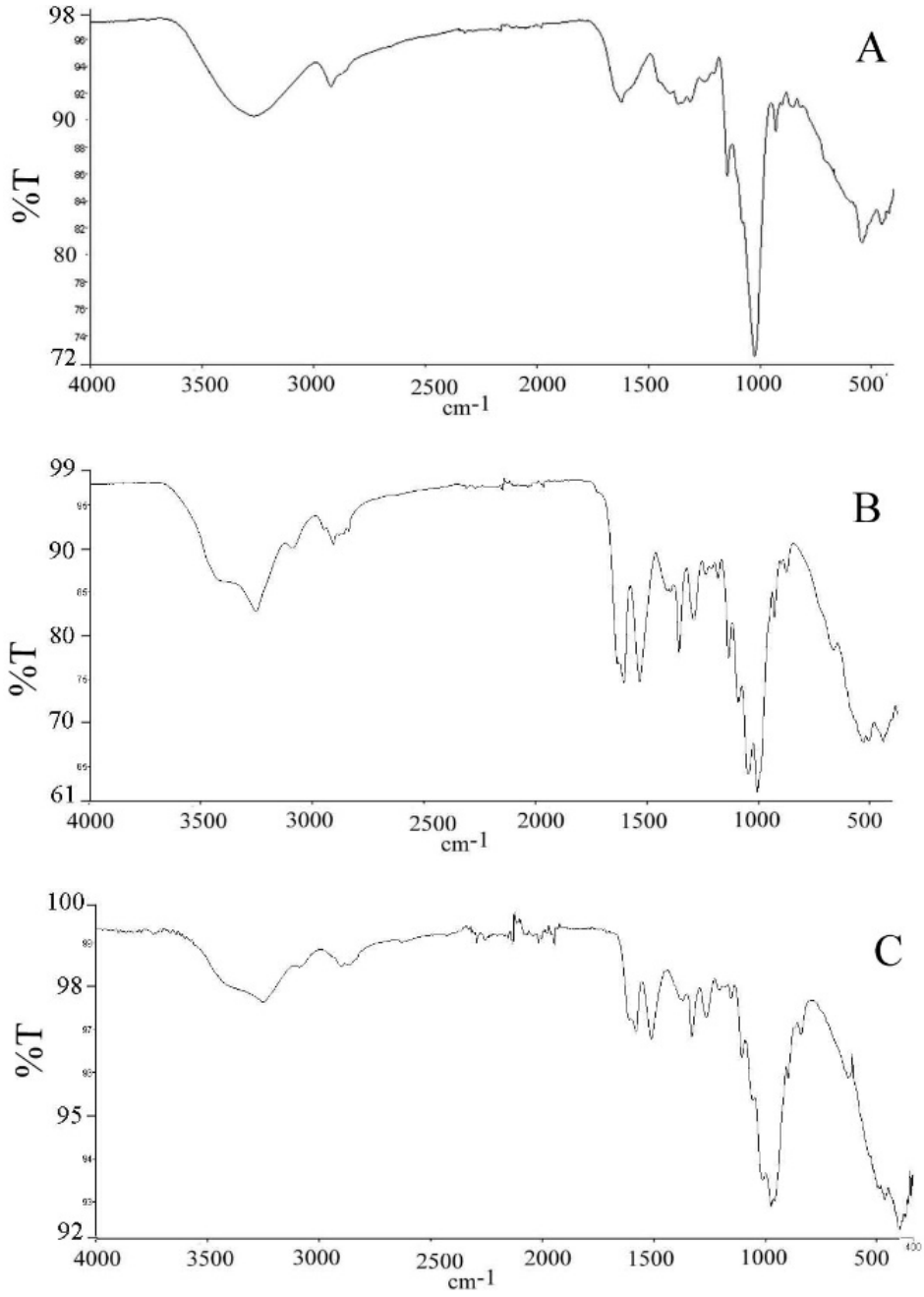


**Figure 1.** Fourier-transform infrared spectra of chitin extracted from fruiting bodies of (A) young, (B) ripe, and (C) overripe *Laetiporus sulphureus*.

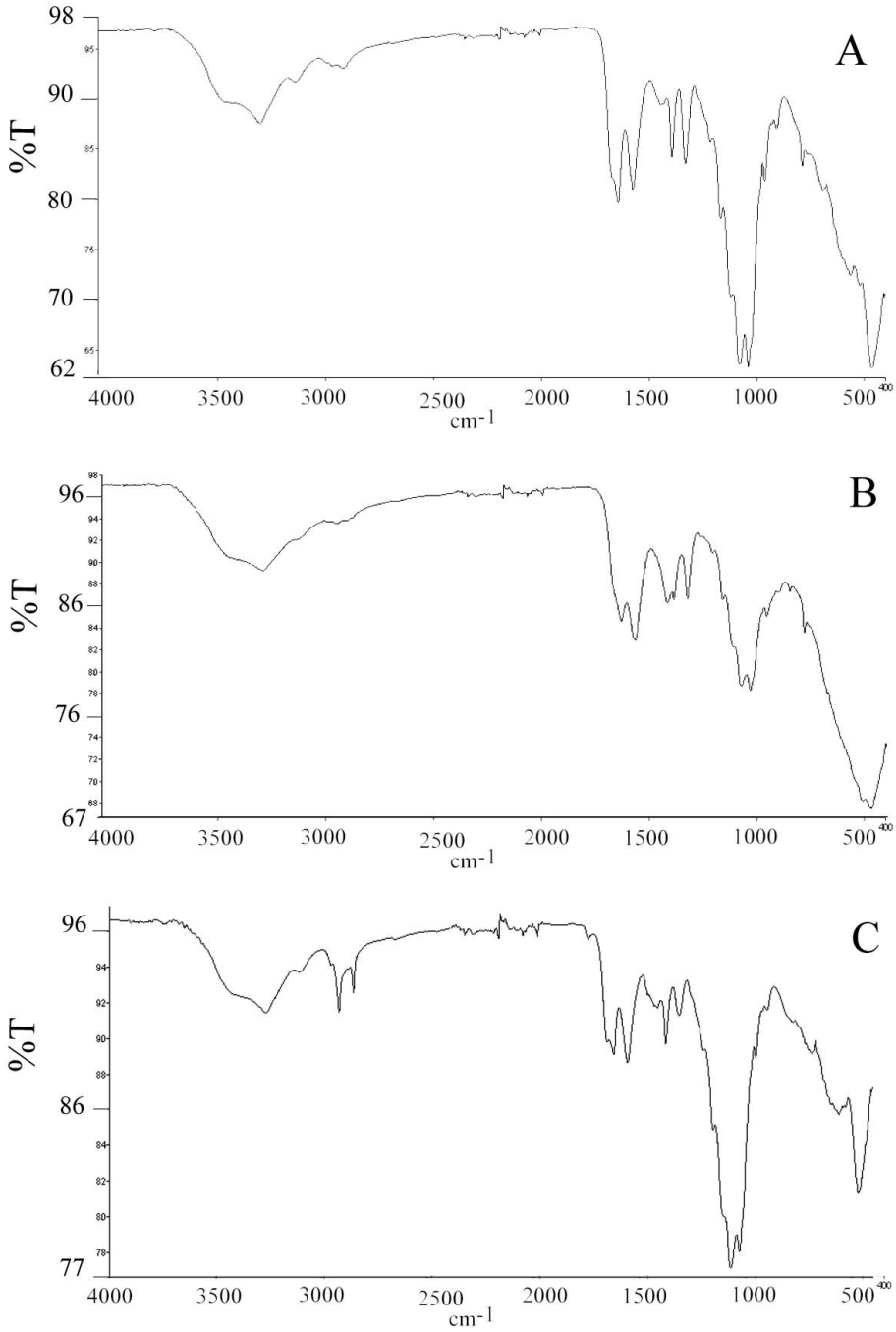


**Figure 2.** Fourier-transform infrared spectra of chitin extracted from fruiting bodies of (A) young, (B) ripe, and (C) overripe *Fomitopsis betulina*.

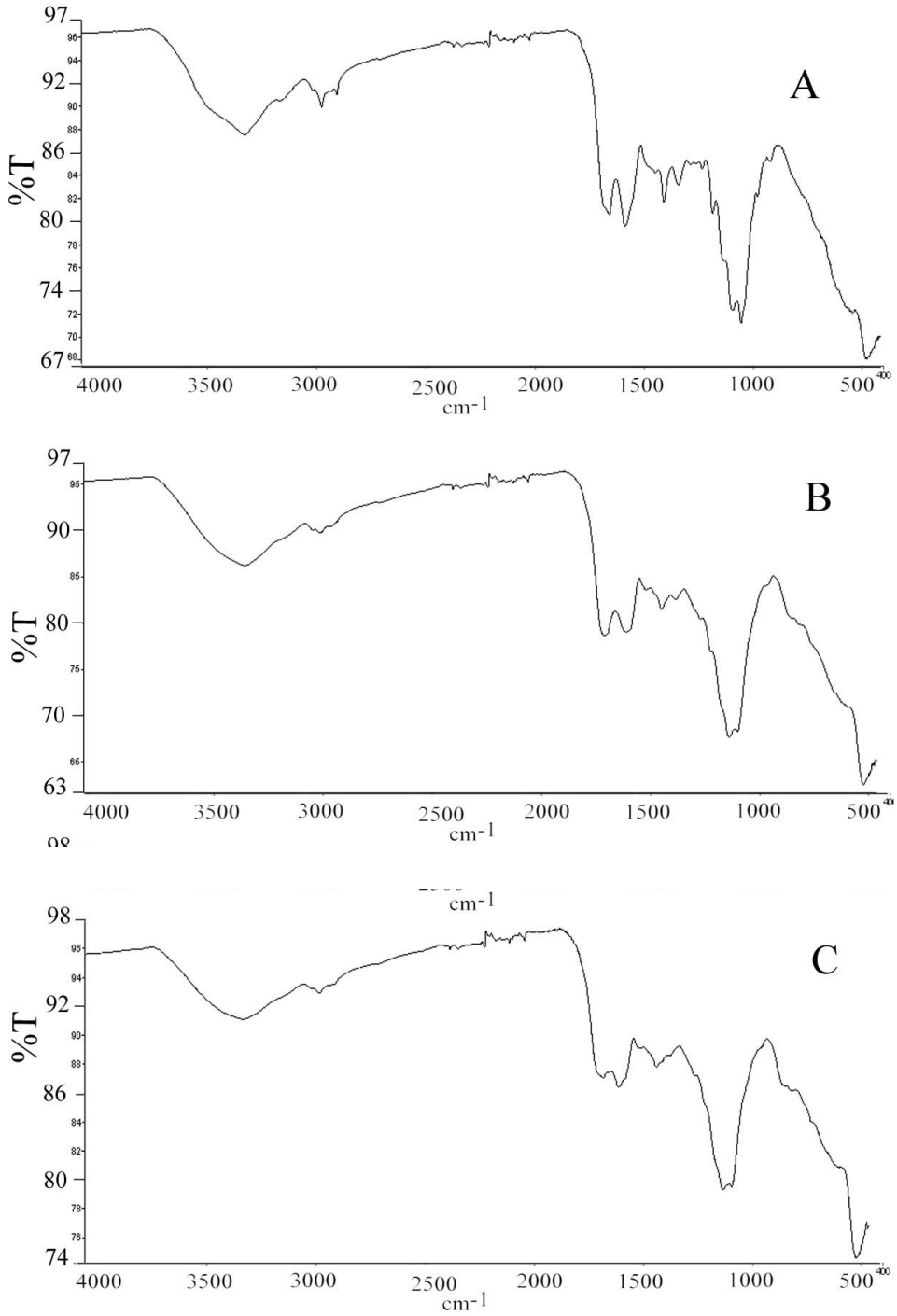


**Figure 3.** Fourier-transform infrared spectra of chitin extracted from fruiting bodies of (A) young, (B) ripe, and (C) overripe *Tyromyces chioneus*.

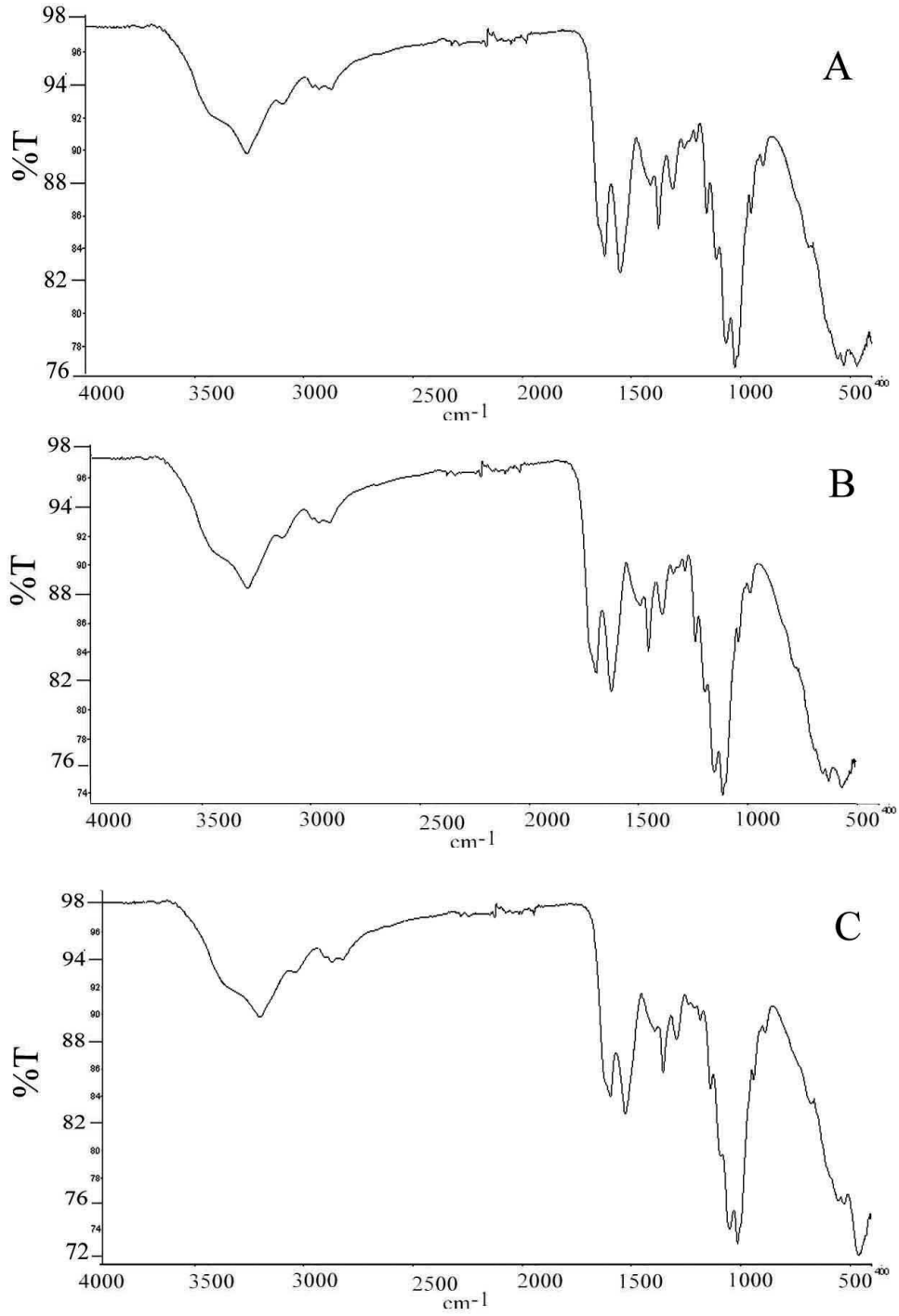


**Figure 4.** Fourier-transform infrared spectra of chitin extracted from fruiting bodies of (A) young, (B) ripe, and (C) overripe *Lycoperdon perlatum*.

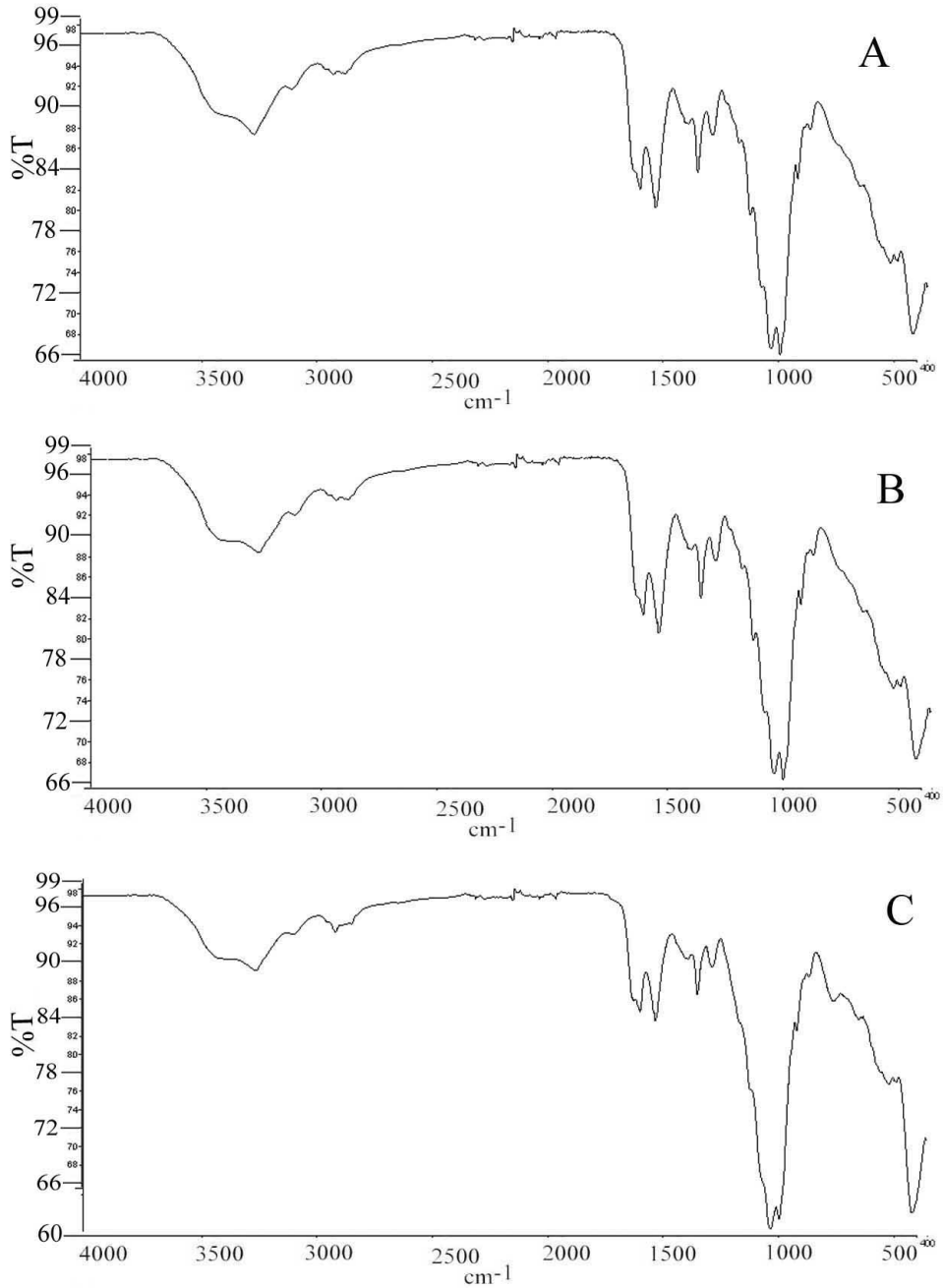


**Figure 5.** Fourier-transform infrared spectra of chitin extracted from fruiting bodies of different stages of (A) young, (B) ripe, and (C) overripe *Oudemansiella mucida*.

Chitin extracted from fruiting bodies with concentrated HCl contains a pigment that is not completely removed even after re-dissolution. According to the literature, it is covalently decorated with β–glucan [15], and possibly melanin, which gives it a dark colour. However, after re-precipitation, chitin became lighter, and its yield was 25%–30% of the originally extracted chitin. According to the literature, the interactions between chitin and glucan

in mushrooms often have a covalent character [15, 16]; therefore, mushroom chitin differs in its properties from crustacean chitin. FT-IR spectroscopy can provide important information regarding purity, the degree of acetylation, and possible impurities. The FT-IR spectra of chitin extracted from fruiting bodies of mushrooms at different stages of maturity are presented in Figures 1–5. The details of the spectra are presented in Table 2.

**Table 2.** Fourier-transform infrared spectra bands [$cm^{-1}$] of chitin extracted from the fruiting bodies of mushrooms.

| *Functional group* | *Shrimp chitin* | *Laetiporus sulphureus* | *Fomitopsis betulina* | *Tyromyces chioneus* | *Lycoperdon perlatum* | *Oudemansiella mucida* |
|---|---|---|---|---|---|---|
| –OH | 3434 | 3418–3434 | 3421–3434 | 3420 | 3424–3434 | 3418–3423 |
| $–NH_2$ | 3256 | 3260–3263 | 3261–3263 | 3264–3273 | 3262–3263 | 3263–3265 |
| $–NH_2$ | 3100 | 3095–3100 | 3100 | 3100 | 3100 | 3100 |
| Aliph. comp. | 2933–2959 | 2918–2961 | 2917–2919 | 2914–2951 | 2925–2963 | 2922–2924 |
| Aliph. comp. | 2876–2890 | 2849–2891 | 2849–2877 | 2849–2873 | 2873–2876 | 2855–2875 |
| Amide I | 1654 | 1650–1653 | 1655 | 1637–1650 | 1650–1653 | 1653–1655 |
| Amide I | 1619 | 1618–1622 | 1619–1621 | 1617–1621 | 1619–1622 | 1621 |
| Amide II | 1552 | 1545–1553 | 1554–1555 | 1535–1549 | 1550–1553 | 1555–1556 |
| –CH | 1413–1428 | 1410–1435 | 1407–1413 | 1446–1454 | 1413–1415 | 1423 |
| –CH | 1375 | 1375–1376 | 1376–1379 | 1372–1375 | 1375 | 1376 |
| Amide III | 1307 | 1308–1313 | 1314–1315 | 1308–1312 | 1309–1311 | 1313 |
| C–O–C | 1069 | 1063–1068 | 1065–1070 | 1064–1068 | 1064–1066 | 1065 |
| C–O | 1008 | 1008–1026 | 1026–1028 | 1026–1028 | 1025–1026 | 1026–1027 |

*Note.* Aliph. comp., aliphatic compounds.

The FTIR spectra confirmed that the 3434, 3330–3130, 1619, 1552, 1375, and 1307 $cm^{-1}$ bands characteristic for chitin are attributed to O–H stretching; N–H stretching; and amide I, II, III, respectively. All these bands are present and similar in the chitin extracted from fruiting bodies of each mushroom species. We also noted the following: a broad band with a maximum at 3433 (O–H stretching); bands at 3330–3130 and 3130–3040 $cm^{-1}$ (N–H stretching); bands at 2953, 2932, 2890, and 2876 $cm^{-1}$ (C–H aliphatic compounds); bands at 1654 and 1619 $cm^{-1}$ (C=O secondary amide stretch; amide I); a band at 1552 (N–H bend, C–N stretch; amide II); bands at 1428, 1413, and 1375 $cm^{-1}$ ($CH_2$ bending); a band at 1307 $cm^{-1}$ ($CH_2$ wagging; amide III); a band at 1154 $cm^{-1}$ (C–O stretching); bands at 1113 and 1069 $cm^{-1}$ (C–O in saccharide rings); band at 1008 $cm^{-1}$ (C–O); a band at 952 $cm^{-1}$ ($CH_3$ wagging);and a band at 894 $cm^{-1}$ (C–H in saccharide rings).

Based on the FT-IR spectra, we can conclude that there is a lot of similarity in the chitin extracted from mushrooms and commercially available shrimp chitin regarding the bond types and chemical composition. We also observed that the wide absorption band around 1530–1560 $cm^{-1}$, which is attributed to a protein band (amide II), is in all chitin extracted from mushrooms. However, this band is quite sharp in the FT-IR spectrum of chitin from shrimp. Those adsorption bands can be assigned to melanin. According to the literature, melanin can be covalently bound to the amino group of *N*–acetyl–D–glucosamine. The precursor of different types of melanin are amino acids [17]. The biological role of melanin is to absorb ultraviolet light and to suppress the formation of free radicals and thus to prevent DNA damage.

According to the literature, *Aphyllophorales* spp., which are xylophagous, wood-rotting fungi, have a lower chitin content. Other species of the orders Boletales, Agaricales, and Russulales have a higher chitin content in their fruiting bodies (8%–9% of dry weight) [2]. The fungi we evaluated in this study belong mainly to the order Aphyllophorales. We studied these mushrooms as a potential source of squalene and steroid substances. We considered the possibility of using the extracts that remained after extracting the lipophilic, alcohol-soluble, and water-soluble substances for the production of chitin. Apparently, only with the complex use of the raw material of the fruit bodies of these fungi, obtaining chitin is expedient. Based on our results and considering our previous study [7], alkaline hydrolysis of the chitin-containing material of mushroom fruiting bodies with the subsequent production of chitosan is a more rational approach. Although extraction with concentrated HCl, a direct method to obtain chitin from a practical point of view, it is nevertheless impractical due to the high risk of chitin hydrolysis, which inevitably increases its loss.

## 4. Conclusions

We obtained chitin from fruiting bodies of *L. sulphureus*, *T. chioneus*, *O. mucida*, *L. perlatum*, and *F. betulina* collected at different stages of maturity. In all cases, the mass of chitin obtained by extraction with 25% HCl increased from young to mature mushrooms, and then decreased after the end of their growing season. The molecular weight of chitin in these samples tended to increase. However, obtaining chitin by extraction with 25% HCl is impractical due to the high risk of chitin hydrolysis, which inevitably increases its loss. When using mushroom fruiting bodies to obtain numerous compounds, from a practical point of view, it is more rational to obtain chitosan by alkaline hydrolysis.

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